Affymetrix®

Procarta® Cytokine Assay Kit

User Manual specifically for Cell Culture Supernatant Samples

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Affymetrix, Inc.

Procarta Cytokine Assay Kit User Manual

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Citing Procarta in Publications

When describing a procedure for publication using this product, we would appreciate it if you would refer to it as the Procarta® Cytokine Assay Kit.

If a paper cites a Procarta product and is published in a research journal, the lead author(s) may receive a travel stipend for use at a technology conference or tradeshow by sending a copy of the paper to our technical support group at techsupport@panomics.com or via fax at (510) 818-2610.

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About the User Manual

Who Should Read this Manual

Anyone that has purchased a Procarta® Cytokine Assay Kit from Affymetrix to perform quantitative, multiplexed cytokine measurements from **Cell Culture Supernatant** samples using the Luminex® Technology.

IMPORTANT Please note that we provide additional application specific manuals for the Procarta assays for the following sample types:

- **Serum/Plasma**
- **Tissue or Cell Lysates**
- **Bodily Fluids**

Our most updated manuals can be obtained from our website at www.panomics.com

What this Manual Covers

This manual provides recommendations and step-by-step procedures for the following:

- **Sample Procedure**
- **Standard Preparation**
- **Assay Procedure**
- **Troubleshooting**

Safety Warnings and Precautions

! WARNING ! Though the general procedure is very similar to other Luminex cytokine assays, there are subtle differences due to the approach we have taken in developing the assay to have an improved workflow. Please do not refer to other vendors' manuals for performing the assay. Please read the supplied User Manual and Product Insert prior to starting the assay.

CAUTION All chemicals should be considered potentially hazardous. We recommend that this product and its components be handled by those trained in laboratory techniques and be used according to the principles of good laboratory practice.

CAUTION This kit contains small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. When disposing, flush drains with a large volume of water to prevent azide accumulation. Observe all state and local regulations for disposal.

Contacting Affymetrix

For technical questions, please contact our technical support group by telephone at 1-877-726-6642 option 3, or email at techsupport@panomics.com (US and Canada). In Europe, contact techsupport_europe@panomics.com. In Asia/Pacific, contact techsupport_asia@panomics.com. For an updated list of FAQs and product support literature, visit our website at www.panomics.com.

About the Procarta Cytokine Assay Kit

Procarta Cytokine Assay Defined

The Procarta Cytokine assays are multiplex immunoassays based on xMAP® detection technology developed by Luminex. This bead based multiplex assay kit can quantitatively measure multiple cytokines from as little as 25 µL of Cell Culture Supernatant samples in less than 4 hours. Procarta Cytokine kits can be used for up to 6 months from the date of receipt.

Available Kit Formats

Procarta Cytokine Assay Kits are available as:

- Standard pre-mixed panels
- "By Request" user configured panels

Procarta Cytokine Assay Kits are available as single or ten plate 96-well formats and contain all the reagents required to detect cytokines.

Procarta Cytokine Assay Kit Contents and Handling Conditions

Kit Contents and Storage

The Procarta Cytokine Assay Kit contains the following components. The kits are available as single plate or ten plate formats. Refer to the product insert for quantities and details of components supplied.

Procarta Cytokine Kit components:

Component	Storage
Reading Buffer	4°C
10X Wash Buffer	4°C
Detection Antibody, premixed	4°C
Antigen Standards, premixed, lyophilized	4°C
Streptavidin-PE (SAPE)	4°C
Antibody Beads	4°C
Filter Plate	4°C
Plate Seals	4°C
PCR 8 Strip Tube	4°C

Kit Handling

- Store the entire kit at 4°C
- Do not reuse or store resuspended antigen standards
- 10X Wash Buffer and its 1X dilution can be stored at 4°C or room temperature for up to 6 months

Required Materials and Equipment Not Provided

Equipment

Item	Source
Vacuum filtration system	Millipore (P/N MAVM0960R and WP6111560)
Microplate shaker	Labline model 4625 or equivalent (must have 3 mm orbit with ability to maintain 500 rpm)
Luminex or Luminex-based instrument	MiraiBio, Bio-Rad or other Luminex instrument provider

Overview of Assay Workflow

Step 1. Prepare Samples and Reagents

a. Collect and prepare samples
b. Prepare antigen standards
c. Prepare 1X Wash Buffer

Step 2. Pre-wet Filter Plate

a. Let sit for 5 min
b. Filter

Step 3. Add Antibody Beads

a. Filter
b. Wash once
c. Filter

Step 4. Add samples/standards

a. Incubate 60 min shaking, 20-25° C
b. Wash/filter three times

Step 5. Add Detection Antibody

a. Incubate 30 min shaking, 20-25° C
b. Wash/filter three times

Step 6. Add Streptavidin-PE

a. Incubate 30 min shaking, 20-25° C
b. Wash/filter three times

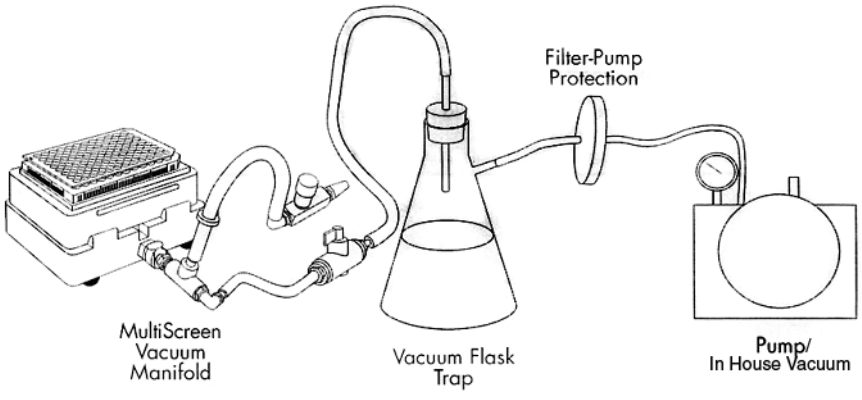
Step 7. Read plate

Getting Started

- **Thoroughly read the Product Insert that is included with your kit. The product insert includes the bead regions and may contain special instructions for proper use of your "By Request" or custom panel.**
- Turn on the Luminex machine and initiate the startup protocol. It takes 30 minutes for the laser to warm-up.
- Vortex the 10X Wash Buffer to dissolve all the salts. Mix 20 mL of the 10X Wash Buffer with 180 mL deionized, sterile water. 1X Wash Buffer can be stored at 4 °C or room temperature for up to 6 months.
- Ensure that the Millipore vacuum manifold has been properly setup and calibrated.
- Change the pipet tips after every transfer and avoid creating bubbles when pipetting.
- During the incubation steps, cover the Filter Plate with aluminum foil to prevent photobleaching of the fluorescent beads.
- Be careful not to invert the plate or allow contents from one well to mix to another well.
- We recommend using a multi-channel pipette whenever possible to reduce CVs.
- Bring all buffers to room temperature before setting up the assay. Store the detection antibody, antibody beads, Streptavidin-PE, Samples, and reconstituted standards on ice during the assay.
- When sealing the Filter plate with a Plate Seal, gently press your finger over the Plate Seal. Too much pressure can cause force the fluid through the filter plate. To avoid Filter Plate leakages, do not seal Filter Plates using a rubber roller (or equivalent) as they apply significant pressure resulting in leakage.
- Seal all unused wells with an enclosed Plate Seal to ensure proper vacuum pressure.

Setting Up and Calibrating the Manifold

To set up and calibrate the manifold:

Step	Action
1	Set up the Filter Plate vacuum manifold as shown below. Follow the manufacturer's manual for details. 

To set up and calibrate the manifold: *(continued)*

Step	Action
2	Calibrate the vacuum pressure using a used Filter Plate: a. Place the Filter Plate on top of the manifold. b. Turn on the vacuum. c. Press down on all 4 corners of the Filter Plate to form a tight seal. d. Adjust the pressure so that it takes 8-10 seconds to evacuate 200 µL of wash buffer from the wells. If the vacuum is too high, beads can get trapped or pulled through the filter. e. Turn off vacuum as soon as the solution filters through the wells and remove the plate from the manifold.

Operating the Manifold

To operate the manifold:

Step	Action
1	Check vacuum calibration periodically. As a general guideline, 200 µL of solution should take approximately 8-10 seconds to clear the well of a Filter Plate.
2	For all filtration steps, turn on the vacuum pump, place the Filter Plate onto the vacuum manifold and then filter the solution. Avoid splashing and cross-contamination of wells during all wash steps. **IMPORTANT** Do not allow the Filter Plates to air-dry following washes. Immediately add the next component after each filtration step. **IMPORTANT** We recommend performing all the wash steps next to the manifold to minimize the amount of time that the beads are exposed to air.
3	Break the vacuum immediately after each solution has been completely filtered from all wells (approximately 8-10 seconds). **Note** Wells may filter at different rates.
4	Following the last wash in each series, blot the bottom of the Filter Plate thoroughly with a paper towel to remove traces of 1X Wash Buffer. Avoid touching the bottom of the Filter Plate with your fingers or to the bench during manipulations. Immediately move to the next step to ensure that the beads are in the appropriate buffered solution.

Sample Preparation

Preparing Samples

- For optimal recovery and sensitivity, it is important to properly and consistently prepare samples.
- Store all samples on ice after preparation and use immediately or aliquot and store at –80°C. Avoid more than 2 freeze/thaw cycles.
- If you are using frozen samples, thaw completely on ice and mix well prior to use.
- Use fresh cell culture media (that is used to culture the experimental cells) to dilute the standards and samples.
- A total volume of 50 µL/well of sample is needed and a minimum of 2 replicates are recommended.

Dilution for High Expression Cytokines

Sample dilution may be needed if the expression of those targets are outside the measurable range. If dilution is needed, use the cell culture media that was used to culture that particular cell line. However, most samples can be used directly in the assay for all other available targets.

Special Sample Preparation for TGF beta

TGF beta has a special protocol for sample preparation. The TGF beta assay can only detect the active form. The complexed form present must be acid treated and then neutralized converting the complexed form to the active form. The assay should be processed as a single plex assay since the sample must be acid treated.

To access our most recent protocols, please visit our website at www.panomics.com.

Standard Preparation

Options for Standard Curve

This section provides two different options for the creation of the standard curve. Depending upon your application, we provide two instruction sets for creating an 8 point standard curve that either ranges from 40,000 pg/ml to 2.44 pg/ml or from 20,000 pg/ml to 1.22 pg/ml.

IMPORTANT Prior to preparing your standard curve, please thoroughly read your product insert. Special instructions for preparation of the standard antigens for some "By Request" panels may be included on your product insert.

Option 1

Option 1: 40,000 pg/ml to 2.44 pg/ml

Step	Action
1	Prior to preparing the standards, please ensure that the Luminex machine has been turned on and calibrated. The laser will take 30 minutes to warm up.
2	Add 250 µL of cell culture media to the lyophilized antigen vial.
3	Vortex gently for 10 seconds and incubate on ice for **25** minutes. Transfer the entire contents of the reconstituted antigen into the first tube of the PCR 8-Strip Tube provided. Use of any other tube other than the Strip Tube provided may result in poor standard recovery.
4	Prepare serial dilutions of the premixed standard as indicated below using the 8 Strip Tube provided: a. Add 150 µL of the cell culture media to tubes 2-8. Follow the table below to create the dilutions. Use a P200 pipette to transfer and mix the 50 µL of standard from tube to tube. b. Store on ice until ready to use. When transferring the standards to the filter plate, we recommend using a multi-channel pipette.
5	Proceed to Performing the Assay

Strip Tube #	Cell Culture Media (µL)	Antigen Standard (µL)	Final Analyte Concentration in the Assay Well (pg/mL)
1	—	250	40,000
2	150	50 from Tube 1	10,000
3	150	50 from Tube 2	2500
4	150	50 from Tube 3	625
5	150	50 from Tube 4	156
6	150	50 from Tube 5	39
7	150	50 from Tube 6	9.8
8	150	50 from Tube 7	2.44

Blank Well	50 µL per well	-	0

Option 2 Option 2: 20,000 pg/ml - 1.22 pg/ml

Step	Action
1	Prior to preparing the standards, please ensure that the Luminex machine has been turned on and calibrated. The laser will take 30 minutes to warm up.
2	Add 500 µL of cell culture media to the lyophilized antigen vial.
3	Vortex gently for 10 seconds and incubate on ice for **25** minutes. Transfer 250 µL to the first tube of the PCR 8-Strip Tube provided and store Strip Tubes on ice. Use of any other tube other than the Strip Tube provided may result in poor standard recovery.
4	Prepare serial dilutions of the premixed standard as indicated below using the 8 Strip Tube provided: a. Add 150 µL of the cell culture media to tubes 2-8. Follow the table below to create the dilutions. Use a P200 pipette to transfer and mix the 50 µL of standard from tube to tube. b. Store on ice until ready to use. When transferring the standards to the filter plate, we recommend using a multi-channel pipette.
5	Proceed to Performing the Assay

Strip Tube #	Cell Culture Media (µL)	Antigen Standard (µL)	Final Analyte Concentration in the Assay Well (pg/mL)
1	—	250 from 500	20,000
2	150	50 from Tube 1	5000
3	150	50 from Tube 2	1250
4	150	50 from Tube 3	312.5
5	150	50 from Tube 4	78.12
6	150	50 from Tube 5	19.53
7	150	50 from Tube 6	4.88
8	150	50 from Tube 7	1.22
Blank Well	50 µL per well	-	0

Performing the Assay

To perform the assay:

Step	Action
1	A blank layout is provided in the Appendix for recording your plate layout. Columns 1 and 2 are typically used for duplicate standards. Mark the sample and blank wells you want to use.
2	Seal the un-used wells of the plate with a Plate Seal prior to starting the assay.
3	Pre-wet the Filter Plate: a. Add 150 µL of Reading Buffer to each unsealed well. b. Incubate 5 minutes at room temperature. c. Remove the Reading Buffer with vacuum filtration. Reading Buffer should clear wells within 8-10 seconds. If outside this range, see “Setting Up and Calibrating the Manifold” on page 9. **IMPORTANT** Do not invert the plate during the rest of this procedure.
4	Add the Antibody Beads: a. Vortex the premixed Antibody Beads for 30 seconds at room temperature. b. Add 50 µL of Antibody Beads to each well. c. Remove buffer with vacuum filtration.
5	Wash the Antibody Beads: a. Add 150 µL/well of 1X Wash Buffer and remove with vacuum filtration. b. Blot the bottom of the Filter Plate thoroughly with paper towels to remove residual buffer.
6	Add 50 µL/well of standards and samples as marked on the plate layout sheet. For the blank samples add 50 µL/well of tissue culture media. Seal the plate gently using a Plate Seal.
7	Incubate and wash the Filter Plate: a. Completely wrap the Filter Plate with aluminum foil and shake at 500 rpm for 60 minutes at room temperature. Optionally, if you decide to incubate overnight, you can shake the plate at room temperature for 30 minutes, transfer the plate to 4°C and store on a level surface. After overnight incubation, remove the plate from 4°C and shake for 30 min at room temperature prior to proceeding to the next step. b. Carefully remove the Plate Seal to avoid splashing the plate contents. c. Remove solution with vacuum filtration. d. When washing the plate, we recommend using multi-channel pipette and a large plastic reservoir for the wash buffer. We also recommend stationing the wash buffer reservoir next to the manifold to minimize the amount of time that the beads are exposed to air. e. Wash the plate three times with 150 µL/well of 1X Wash Buffer. After the 3rd wash, thoroughly blot the bottom of the Filter Plate with paper towels.
8	Add premixed Detection Antibodies: a. Add 25 µL/well of the Detection Antibody. b. Seal the Filter Plate with a new Plate Seal. c. Wrap the Filter plate with aluminum foil and shake at 500 rpm for 30 minutes at room temperature.

To perform the assay: *(continued)*

Step	Action
9	Remove the solution with vacuum filtration and wash the plate: a. Carefully remove the Plate Seal to avoid splashing the plate contents. b. Remove solution with vacuum filtration. c. Wash the plate three times with 150 µL/well of 1X Wash Buffer. After the 3rd wash, thoroughly blot the bottom of the Filter Plate with paper towels.
10	Add Streptavidin–PE: a. Vortex the Streptavidin–PE. b. Add 50 µL/well of Streptavidin-PE. c. Seal the Filter Plate with a new Plate Seal. d. Completely wrap the Filter Plate with aluminum foil. e. Shake at 500 rpm for 30 minutes at room temperature.
11	Remove the solution with vacuum filtration and wash the plate: a. Carefully remove the Plate Seal to avoid splashing the plate contents. b. Remove solution with vacuum filtration. c. Wash the plate three times with 150 µL/well of 1X Wash Buffer. After the 3rd wash, thoroughly blot the bottom of the Filter Plate with paper towels.
12	Prepare plate for analysis on a Luminex instrument: a. Add 120 µL/well of the Reading Buffer. b. Seal the Filter Plate with a new Plate Seal. c. Shake at 500 rpm for 5 minutes at room temperature or until the Luminex instrument is available. d. Remove the Plate Seal before analyzing on the Luminex instrument. **Note** The Filter Plate can be wrapped with aluminum foil and stored for up to 48 hours before proceeding. After storage, remove the Reading Buffer using vacuum filtration and add 120 µL/well of Reading Buffer. Shake at 500 rpm for 5 minutes at room temperature prior to reading. Delay in reading the plate may result in decreased sensitivity for some analytes.
13	Please refer to your product insert when entering the bead analyte associations into your designated Luminex acquisition software (Bio-Plex, MasterPlex, xPonent).
14	Please refer to the previous section, “Standard Preparation” step 4, when calculating the concentration of the samples and confirm which option for the standard curve was used. The concentration of the samples can be calculated by plotting the expected concentration of the standards against the MFI generated by each standard. A 4PL or 5PL algorithm is recommended for the best curve fit. If 50 µL of sample was used, then no dilution factor is needed.

To perform the assay: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>15</td><td>Analyze the plate following the respective operation manual for the Luminex or Luminex-based instrument.
<table>
<tr><th>Software</th><th>Sample Size</th><th>DD Gate</th><th>Timeout</th><th>Bead Events/Bead Region</th></tr>
<tr><td>Luminex 100/ IS100 v 2.318</td><td>50 µL</td><td>7,500-15,000</td><td>25 sec</td><td>50 or 100</td></tr>
<tr><td>Bioplex/ Bio-Rad</td><td>50 µL</td><td>4335-10,000</td><td>45 sec</td><td>50 or 100</td></tr>
<tr><td>Miraibio/ Hitachi</td><td>50 µL</td><td>2,000-15,000</td><td>25 sec</td><td>50 or 100</td></tr>
<tr><td>Starstation/ ACS</td><td>50 µL</td><td>4,335-10,000</td><td>25 sec</td><td>50 or 100</td></tr>
</table></td></tr>
<tr><td>16</td><td>When the Luminex beads are injected into the flow cell, a small percentage of the beads will have a tendency to clump and go through the flow cell as doublets. The DD Gate or Doublet Discriminator Gate will allow for discrimination of the doublets from the singlet beads. When setting the DD Gate, you can follow the appropriate settings from the table above. However, for the best results, you should adjust the DD Gate and center the gates around the largest peak which is the singlet beads. You can adjust the gates when processing the first sample.

The Bio-Plex® Suspension Array System allows calibration using Low or High sensitivity settings. Perform the sensitivity selection during calibration, using predetermined values of CAL2 RP1 target, as provided by Bio-Rad. Using the RP1 Low target value will provide results comparable to those obtained from the Luminex 100. Using the RP1 High target value may increase detection sensitivity for low cytokine protein concentrations but sacrifices part of the linear dynamic range for high concentrations. We recommend RP1 Low target value for BioPlex.

The number of events per region can be 50 or 100. Please note that reading 100 events will take longer to read the entire plate.

IMPORTANT Check to ensure the probe height in the Luminex instrument is adjusted appropriately for the Filter Plate.

IMPORTANT We recommend that you calibrate the Luminex or Luminex-based instrument each day the assay is run.

IMPORTANT If there is a malfunction of the instrument or software during the run, the plate can be re-processed on the Luminex machine. Remove the plate from the instrument and remove the Reading Buffer by vacuum filtration. Resuspend the beads in 120 µL of Reading Buffer and shake at 500 rpm for 5 minutes at room temperature. The plate may take longer to read since there will be less beads in the plate.</td></tr>
</table>

Troubleshooting

Possible Problems and Recommended Solutions

Observation	Possible Cause	Recommended Action
Filter plate Leakage or	Vacuum pressure too high	Adjust the vacuum pressure as recommended in “Setting Up and Calibrating the Manifold” on page 9.
	Filter Plate misaligned (at an angle) during incubation or processing steps	Set the Filter Plate on a flat, level surface during incubation/processing.
	Leakage from capillary action	After each vacuum step, thoroughly blot the bottom of the Filter Plate using paper towels or absorbent paper.
	Too much force applied to the Plate Seal	Apply a Plate Seal onto the plate and gently press using your index finger.
Filter plate is clogged	Samples clogging the filter plate	Further dilute the samples prior to adding to the plate.
High CVs	Samples and antigen standards not stored on ice	Prepare the samples and standards on ice before setting up the assay.
	Bottom of the Filter Plate is not dry	After each vacuum step, thoroughly blot the bottom of the Filter Plate using paper towels or absorbent paper.
	Contamination from re-using the Plate Seal	Use a new Plate Seal for each incubation step.
	Incomplete washing	After adding the standards and samples, it is very important that any excess standards are removed during the wash step.
	Contamination from Wash Buffer	Be careful not to splash Wash Buffer during wash steps into adjacent wells.
	Pipetting techniques	Using single channel pipettes and poor pipette techniques can cause high CVs especially towards the lower end of the dilution curve.
Limited dynamic range when using the BioPlex software	Instrument calibrated at high PMT settings	Calibrate the instrument using the CAL2 Low RP1 target value.

Observation	Possible Cause	Recommended Action
Low bead count	Volume of bead solution is too low	Add 120 µL/well Reading Buffer and shake at 500 rpm for 5 minutes at room temperature to resuspend beads before reading in the Luminex instrument
	High bead aggregation	Vortex the bead solution well before using in the assay and ensure that the beads are properly mixed during the incubation steps
	Vacuum pressure too high	Confirm that it takes 8-10 seconds to evacuate 200 µL of wash buffer
	Filter ruptured due to excess vacuum time	Do not use the vacuum over 10 seconds in any of the steps
	Dyes contained in the beads are photo-bleached from overexposure to light	Store bead solution and the assay plate in the dark
	Samples causing the instrument to clog	May need to dilute the sample or stop and shake the plate in the middle of the run
	Probe height is incorrect	Refer to the Luminex manual for proper adjustment of the needle height
	Air bubble in the sample loop	Refer to the Luminex manual for proper removal of the air bubble
Low signal or sensitivity	Standards not reconstituted and diluted correctly	Prepare fresh antigen standards following the instructions in “Standard Preparation” on page 12
	Beads stuck to the bottom of the plate	Confirm that the plate shaker is set to 500 rpm and shaking for at least 5 minutes before reading
Poor recovery	Did not use appropriate cell culture media to prepare the standards.	Use the same cell culture media that was used to culture the cells
	Samples and antigen standards were not stored on ice	Prepare the samples and standards on ice before setting up the assay and let set for 25 minutes

Appendix

Limit of Detection

The limit of detection (LOD) is the lowest analyte concentration that can be detected in a sample but not necessarily quantified. LOD is based on the Mean Fluorescence Intensity (MFI) generated by the Luminex instrument when measuring the assay reporter molecule (SAPE) on the multi-fluorescent coded microspheres. The MFI and the Standard Deviation (SD) of the assay blank is used to calculate the LOD. To measure the LOD of an assay, the SD is multiplied by 2 and added to the MFI value of the assay blank. If that total calculated value is lower than the lowest standard, then the LOD would be considered lower than the lowest standard. The calculated LOD can be measured by creating a linear curve fit of the lowest 3 standards and then back-calculating the concentration of the assay blank using that linear curve fit equation. LOD can be measured when the standards are prepared in at least duplicate. Most cytokine assays have a LOD less than 1 pg/ml when measured in cell culture supernatants samples and less than 10 pg/ml when measured in serum or plasma.

Limit of Quantitation

The lower limit of quantitation (LLOQ) is the lowest analyte concentration that can be quantified with acceptable precision and accuracy. The upper limit of quantitation (ULOQ) is the highest analyte concentration that can be quantified with acceptable precision and accuracy. The LLOQ and LLOQ are expressed in concentration units such as pg/ml. The algorithms used to create the standard curves will impact the LOQ range. Depending upon the shape of the curve, a 5 parameter logarithmic curve fit (5PL) may yield better accuracy compared to a 4 parameter logarithmic curve fit (4PL). The standard curve is generated using 6 to 9 standards with increasing levels of known standard antigens diluted in an appropriate standard matrix. The actual MFI versus the known concentration of standards is plotted and a 4PL or 5PL algorithm is used to generate a curve. This 4PL or 5PL curve fitting equation can calculate the concentration of unknown samples using MFI as the input value. When the MFI of the standard value is entered into the curve fit equation as an unknown, the concentration value that is generated from the curve fit equation can be used to evaluate the standard recovery. The standard recovery is calculated by taking the ratio of this calculated concentration value divided by the expected amount of standard and expressing that as a percentage. An acceptable range of recovery should be between 70-130%. In addition, the LLOQ must be higher than the limit of detection. The LLOQ for most cytokine assays in cell culture supernatant, serum and plasma samples is less than 20 pg/ml.

Accuracy/ Recovery

Accuracy is the closeness of test results obtained by the analytical method to the true value. Accuracy is reported as the percent recovery of known added amounts of analyte in the sample matrix. Percent recovery can be expressed relative to the targeted spike concentration of analyte added to biological matrix. Accuracy should be assessed using a minimum of 6 determinations over a minimum of 3 concentration levels (e.g. 3 concentrations/ 2 replicates) in a representative pool of sample matrix (preferably the same pool of matrix used to prepare matrix controls). The analyte concentrations tested should be targeted to the same region of the standard curve as the matrix controls. In general, accuracy should be within the range of 70 - 130% recovery of exogenous analyte.

Precision

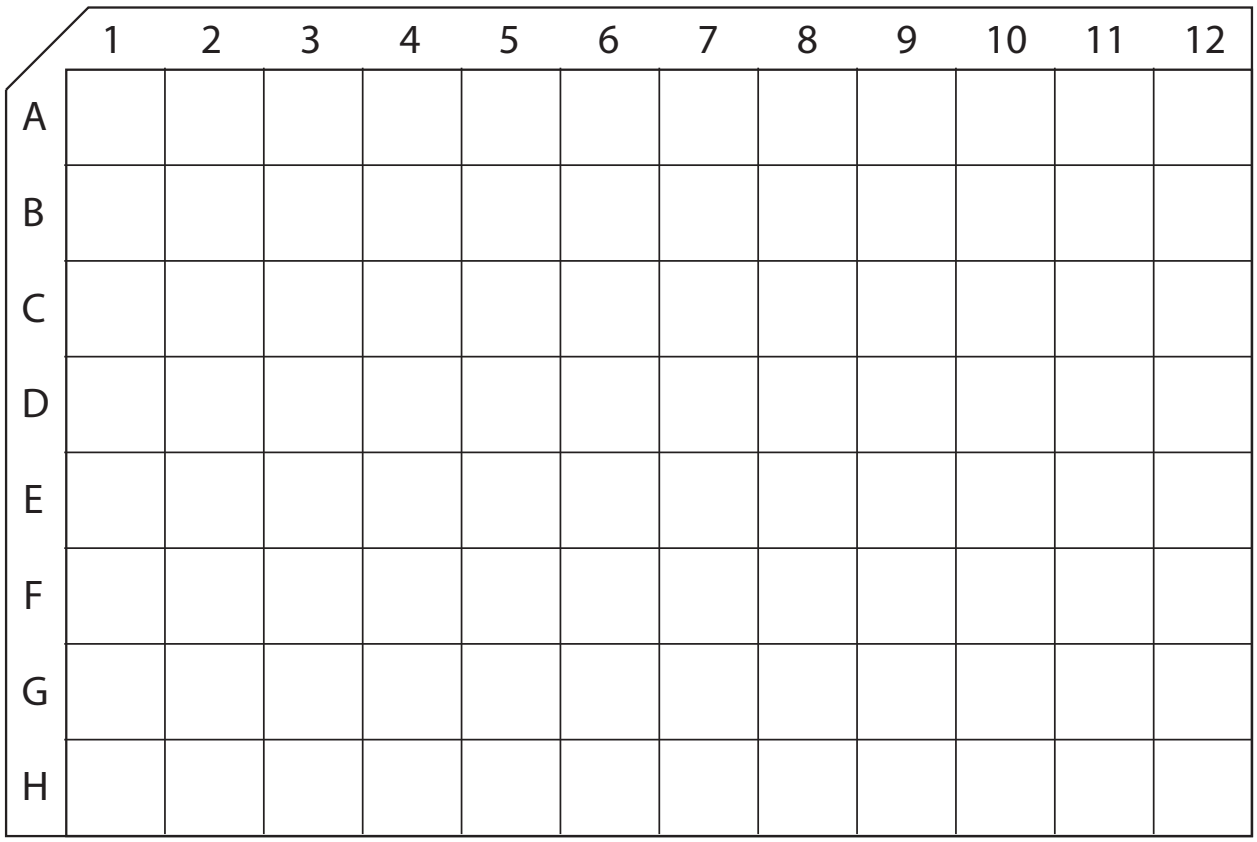
Precision is the degree of agreement among individual test results when the analytical method is repeated for multiple measurements of a sample. Intra- and

inter-assay precision should be evaluated from a single series of 10-20 assays with 2-4 replicates of the low, mid, and high matrix controls using a one-way analysis of variance. The precision determined for matrix controls should confirm the LLOQ and ULOQ determined by precision profile analysis. The inter-assay precision determined for matrix controls should be less than 20% CV. For the purpose of evaluating immunoassay precision, a different preparation of the standard curve must be used for each assay. Ideally, precision should include data from different technicians as well.

Specificity Specificity is the ability of an analytical method to accurately measure the analyte in the presence of other sample matrix components. Qualification of assay specificity includes studies of potential cross-reactive molecules as well as molecules that may interfere with analyte quantitation. In our assays, dose-response curves of multiple analytes are measured simultaneously in the presence and absence of antigen standards to assess the cross-reactivity of the antigens and the affinity of antibodies used in the assay. The cross reactivity of our assays is negligible and does not exceed 5%.

Sample and Blank Plate Layouts

	Standards		Samples									
	1 pg/mL	2 pg/mL	3	4	5	6	7	8	9	10	11	12
A	40,000	40,000	1	1	8	8	16	16	24	24	32	32
B	10,000	10,000	2	2	9	9	17	17	25	25	33	33
C	2500	2500	3	3	10	10	18	18	26	26	34	34
D	625	625	4	4	11	11	19	19	27	27	35	35
E	156	156	5	5	12	12	20	20	28	28	36	36
F	39	39	6	6	13	13	21	21	29	29	37	37
G	9.8	9.8	7	7	14	14	22	22	30	30	38	38
H	2.44	2.44	Blank	Blank	15	15	23	23	31	31	39	39



Luminex Maintenance

For optimal results, we strongly recommend that you clean the sample probe/needle on a regular basis according to the manufacturer's recommendations. Probe/needles that have rusty or salt deposits should be replaced (Luminex P/N CN-0007-01).

Clean the instrument after each run as indicated in the following table:

Step	Number of Times	Solution
Sanitize	2X	20% Bleach
Backflush	3X	None
Alcohol Wash	3X	70% Ethanol
Wash	4X	Distilled Water

Clean the instrument when shutting down as indicated in the following table

Step	Number of Times	Solution
Sanitize	2X	20% Bleach
Backflush	3X	None
Alcohol Wash	3X	70% Ethanol
Wash	4X	Distilled Water
Soak	1X	Water